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# Ultrasensitive Biosensors for Molecular Recognition and Manipulation

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## Abstract

Our objective is to develop novel biomolecule recognition mechanisms and ultrasensitive biosensors for direct, real-time biochemical imaging and sensing. These biosensors will provide a novel tool which permits major advances in the investigation and control of fundamental molecular and cellular physiological processes. There are three aspects of our approach: 1. Using nanotechnology and existing sensing mechanisms for nanometer level biosensor development; 2. Using molecular beacon DNA molecules for development of new biomolecule recognition mechanisms; 3. Using single molecule microscopy techniques for molecular interaction studies. Over the three years of this grant, we have published 20 papers and filed two patents (one granted and one pending). The grant also has helped us to train six graduate students, three postdoctoral researchers, five undergraduate students. Among all the students, there are two African American graduate students, one Hispanic graduate student and three minority undergraduate students. The grant has also enabled us to build a world class research laboratory in the area of biomolecular interaction and recognition studies.

## Research achievements

1. **Development of DNA/RNA biosensors.** We have developed a variety of molecular beacon based DNA/RNA biosensors for ultrasensitive detection. We have prepared single point sensors as well as DNA sensor arrays for DNA/mRNA detection. We have also used nanoparticles for DNA biosensor development. A molecular beacon (MB) is an ssDNA probe for detection of nucleic acids and proteins. A MB possesses a loop and stem structure with a fluorophore and a quencher linked at either end of its stem. Its signal transduction mechanism for molecular recognition is based on fluorescence energy transfer (FRET). The MBs act like switches that are normally closed to bring the fluorophore/quencher pair together to turn fluorescence "off". Upon binding to the target molecules, they undergo conformational changes that open the hairpin structure and separate the fluorophore and quencher, thus turning "on" the fluorescence. This feature makes MBs an extremely useful probe in a variety of applications such as the monitoring of real time PCR processes and the detection and dynamic study of mRNA in living cells.

We have designed biotinylated ssDNA MBs for DNA hybridization studies at a solid surface. The biotinylated MB with tetramethylrhodamine (TMR) as the fluorophore and DABCYL as the quencher has been custom synthesized. This biotinylated MB can be immobilized onto a silica

surface and hybridized with its cDNA. Based on this surface immobilized MB, we have developed different DNA/RNA biosensors: a fiber optical evanescent wave sensor, a submicrometer optical fiber sensor, a nanoparticle based biosensor and biosensor arrays. The sensors are rapid, sensitive, stable, highly selective, reproducible and regenerable. They have been applied to detect specific DNA and mRNA sequences and to the study of DNA hybridization kinetics. The target DNA concentration detection limit of the MB biosensors is in the pico-Molar ( $10^{-12}$  M) range, and the smallest amount of DNA has been detected is in attomoles. Using an array imaging technique, we are able to detect ten DNA molecules. The microwell array was fabricated by photolithographic and wet chemical etching techniques. The volume of each well is about 28 femtoliters. The ultrasmall volume compared to the traditional DNA microarray techniques reduces the cost of sample, reagent and analysis time. We intend to use this array system for the determination of multiple cancer proteins and protein variants and thus for cancer cell proteomics studies with multiple parameters.

**2. Single molecule reaction and interaction:** We have studied single biomolecule interaction and reaction at an interface of liquid/solid. We have investigated single DNA molecule hybridization dynamics using surface immobilizable molecular beacons.

We have developed an ultrasensitive fluorescent microscopy technique for single molecule detection and imaging. The fluorophores were excited by an evanescent wave produced on an optical fiber probe surface. The fluorescent signals were detected by an Intensified CCD camera. Single fluorophore molecules have been imaged. The single molecule imaging capability realized by an optical fiber can be directly adopted for DNA/RNA detection in real-time. We have also directly observed single molecule reaction at a solid-liquid interface. The reaction between one surface immobilized molecule and one flowing molecule in the solution was studied. Time-lapse fluorescence images of single molecule products, excited by the evanescent field produced at a quartz-liquid interface, were recorded to follow the biochemical reaction. This approach offered a novel means to study single molecule interaction and reactions at the solid-liquid interface. It also enables biochemical mapping of surface heterogeneity at the single molecule level. Using this technique, we have also studied single DNA molecule hybridization kinetics using surface immobilizable MB on a silica plate. MB hybridization has been monitored after reacted with its target DNA. We are able to monitor the progression of the surface hybridization. Using this technique, we have studied molecular beacon hybridization dynamics. Better understanding has been achieved regarding the sometimes-high background signal for unhybridized surface bound MBs, and improvements in MB based biosensor development is achieved.

**3. Development of molecular beacon DNA probes for protein recognition:** Using MBs, we have studied nonspecific protein-DNA binding. We have also developed a novel approach for real-time protein detection by combining MB's excellent signal transduction with aptamer's specificity in protein binding. There is no technology available that can either identify or quantify proteins in real time and in a rapid method. MBs were originally developed for the detection of nucleic acids, but we found their promising application in the detection of DNA-binding proteins. We have attempted, using the novel MBs and the excellent biomolecular recognition capability of aptamers, to introduce a new approach for directly monitoring protein production in living cells. We started working with a nonspecific MB for single stranded DNA binding protein studies, continued with detailed protein-DNA binding investigation with enzymes such as lactate dehydrogenase, and then developed a MBA for a specific model protein, thrombin. We further continued this line of research by developing an aptamer MB for PDGF, a growth factor relevant to cancer diagnosis and mechanism studies.

**4. Living cell studies.** Using the tools developed during this grant, we have studied living cells with the biosensors developed above. **a. Single living cell calcium influx upon drug stimulation:** We have utilized a microscope/CCD camera system to follow subcellular events in real time. The effects of a series of reagents, such as NMDA, glutamate and other drugs, on  $\text{Ca}^{2+}$  intracellular influx have been studied. Using near-field optics (NFO) probes and biosensors, we have carried out experiments, with much improved spatial resolution, to study ischemia and stroke at the cellular level. We introduced a NFO probe into live cultured vascular smooth muscle cells (VSMC) and NG108-15 neuroblastoma cells. The inserted probe can monitor  $\text{Ca}^{2+}$  influx through a  $\text{Ca}^{2+}$  dye.  $\text{Ca}^{2+}$  influx was initiated using a drug, Angetisen II. The probes can monitor intracellular events either on a cell's membrane or inside the cell. Our studies of  $\text{Ca}^{2+}$  real-time monitoring have shown the potential of the NFO probe in monitoring ultrasmall regions of a living cell. The results demonstrate that NFO is useful for detecting resting  $[\text{Ca}^{2+}]$  and increases in  $[\text{Ca}^{2+}]$  after drug administration. Various response profiles have been observed among different cells, upon different drug administration. NFO enables the visualization of functional responses in living cells with subwavelength spatial resolution. **b. Real-time monitoring of neurotransmitter release using biosensors.** We have studied single living cell and brain tissue neurotransmitter (glutamate, lactate and GABA) release using optical fiber glutamate and lactate sensors. A glutamate biosensor is positioned next to glutamate releasing neuron cell membrane by a micromanipulator. Glutamate release is induced by NMDA stimulation. Both ischemic-induced neurons and NG-108 neuroblastoma cells are used for glutamate monitoring by the glutamate sensor. Quantitative release of glutamate from single cells is followed. The released glutamate has a quick rise once the cell is stimulated. Neurotransmitter release has also been studied using spatially resolved two-dimensional (2D) imaging biosensors. Interesting results have been obtained with brain tissue samples. Real-time monitoring of lactate release from brain slice has been carried out with a 2D biosensor, which is prepared by direct immobilization of lactate dehydrogenase (LDH) molecules onto a flat silica quartz glass surface through a covalent binding mechanism. The biosensor is able to spatially differentiate lactate concentration variations with conventional optical microscopic spatial resolution. This biosensor has the capability to effectively detect lactate down to a concentration of 100 nM. With a rapid response time, this 2D biosensor is able to monitor many cells simultaneously in one image. Drug induced lactate release has been investigated in real-time. We found that different cellular areas have various amount of lactate released. We have studied the impact of nicotine and KCl on lactate release from the brain slices. The spatially resolved determination of the newly released lactate from the mouse brain slices clearly demonstrates the feasibility of real time imaging of lactate release from living specimen. The 2D biosensor will enable us to study cellular communications and possibly other biological processes that require simultaneous temporal and spatial resolutions.

## Publications

1. Design a molecular beacon with two dye molecules, Peng Zhang, Terry Beck and Weihong Tan, Angewandte Chemie International Edition, 2001, 40, 402-405.
2. Development of novel dye-doped silica nanoparticles for biomarker application, S. Santra, K. Wang, Rovelyn Tapeç and Weihong Tan, J. of Biomedical Optics, 2001, 6 (2), 160-166.
3. Real time monitoring of mRNA hybridization in single living cells, John Perlette and Weihong Tan, Analytical Chemistry, 2001, 73, 5544-5550.

4. Conjugation of Biomolecules with Luminophore-Doped Silica Nanoparticles for Photostable Biomarkers, Swadeshmukul Santra, Peng Zhang, Kemin Wang, Roverlyn Tapeç and Weihong Tan, Analytical Chemistry, **2001**, 73, 4988-4993.
5. Genomics and Proteomics Technologies, Ramesh Raghavachari and Weihong Tan, Editors, SPIE, **2001**, 4264, 256pp.
6. Biochemical functionalization of silica nanoparticles, Monde Qhobosheane, Swadeshmukul Santra, Peng Zhang and Weihong Tan, Analyst, **2001**, 126, 1274-1278.
7. Molecular beacons: detecting protein-nucleic acid interactions, Jianwei Li, X. Fang, Sheldon Schuster and Weihong Tan, Angewandte Chemie Inter. Ed., **2000**, 39, 1049.
8. Direct observation of single molecule generation at a solid-liquid interface, Peng Zhang and Weihong Tan, Chemistry, A European Journal, **2000**, 6, 1087-1092.
9. Subwavelength optical microscopy and spectroscopy using near-field optics, Peng Zhang, Raoul Kopelman and Weihong Tan, Editor: Paul Holloway, CRC in Solid State, June, **2000**, 25, 87-162.
10. Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of ssDNA, Jianwei Jeffery Li, Ron Guyer and Weihong Tan, Nucleic Acids Research, **2000**, 28, 11, e52, 1-5.
11. Ultrasmall and ultrasensitive molecular beacon DNA biosensors, Xiaojing Liu, William Famurie, Sheldon Schuster and Weihong Tan, Analytical Biochemistry, **2000**, 283, 56.
12. Using molecular beacons to probe the interactions between lactic dehydrogenase and single strand DNA, Xiaohong Fang, Jeffery Li and Weihong Tan, Anal Chem, **2000**, 72, 3280-3285.
13. Nanoscopic biochemical sensors and probes, Weihong Tan and Raoul Kopelman, in Handbook of nanostructured materials and nanotechnology, Ed. Hari Singh Nalwa, Academic Press, San Diego, **2000**, 621-667.
14. Designing a novel molecular beacon for surface immobilized DNA hybridization studies, Xiaohong Fang, Xiaojing Liu, Sheldon Schuster and Weihong Tan, Journal of American Chemical Society, **1999**, 121, 2921-2922.
15. Probing intracellular dynamics in living cells with near-field optics, Jonathan D. Bui, Tibor Zelles, Jane Hua Lou, Valerie L. Gallion, M. Ian Phillips and Weihong Tan, Journal of Neuroscience Methods, **1999**, 89, 9-15.
16. A Fiber Optic Evanescent Wave DNA Biosensor Based on Novel Molecular Beacons, Xiaojing Liu and Weihong Tan, Analytical Chemistry, **1999**, 71, 5054-5059.
17. Imaging single fluorescent molecule at the interface of an optical fiber probe using evanescent wave excitation, Xiaohong Fang and Weihong Tan, Analytical Chemistry, **1999**, 71, 3101-3105.

18. Development of an optical fiber lactate sensor, Xiaojing Liu and Weihong Tan, Mikrochimica Acta, 1999, 131, 129-135.
19. Direct immobilization of glutamate dehydrogenase on optic fiber probes for ultrasensitive glutamate monitoring, Julia Cordek, Xinwen Wang and Weihong Tan, Analytical Chemistry, 1999, 71(8), 1529-1533.
20. Ultrasmall optical sensors for cellular measurements, Weihong Tan, Raoul Kopelman, Susan Barker and Michael Miller, Analytical Chemistry, 1999, 71 (17), 606A-612A.

## Patents

21. Method for identifying and labeling biological cells, Weihong Tan, Swadeshmukul Santra, Peng Zhang, Rovelyn Tapeç and Jon Dobson, US Patent, 2002, 10/010,807.
22. Development of novel silica-coated nanoparticles, Weihong Tan, Swadeshmukul Santra, Peng Zhang, Rovelyn Tapeç and Jon Dobson, US Patent, 2001, 09/572,469.

## STUDENTS, RESEARCH ASSOCIATES AND TRAINING

### Graduate Students

- Julia Cordek**, M. Sc., 1996-1998, **Bioanalytical Chemistry**, Fabrication and characterization of novel ultra-sensitive glutamate and GABA optical fiber biosensor probes, December, 1998.
- Jane Hua Lou**, Ph. D, 1997- 2002, **Bioanalytical Chemistry**, Molecular beacon application for genes and protein studies, , 2002.
- John Perlette**, M. Sc., 1998-2001, **Biochemistry**, Living cell nucleic acids determination using molecular beacons, August 2001
- Rovelyn Tapeç**, Ph. D, 1998 - 2002, **Bioanalytical Chemistry**, Engineering functionalized nanostructures and nanoparticles for biosensor development, August, 2002.
- Shannon Kelley**, M. Sc., 1998 - 2001, **Biochemistry and Bioanalytical Chemistry**, DNA/RNA hybridization and DNA/RNA-protein interaction studies, December, 2001.
- Monde Qhohobsheane**, Ph. D Candidate, 1998, 6 - now, **Bioanalytical Chemistry**, Two dimensional biochemical sensors and nanoparticles and investigation of glutamate-calcium assumption in ischemic stroke, on-going.
- Lisa Hilliard**, Ph. D candidate, 1999 - now, **Bioanalytical Chemistry**, Bionanotechnology: analytical development for bacterium analysis, on-going.
- Bernd Liesenfeld**, Ph. D candidate, 1997 - now, **Biomedical Engineering**, Engineering an optical patch-clamp for single ion channel recording, on-going.
- Zehui Cao**, Ph. D pre-candidate, 1999 - now, **Bioanalytical Chemistry**, Molecular design and anisotropy imaging, on-going.
- Karina Kerns**, M. Sc. candidate, 1999, 6 - now, **Bioanalytical Chemistry**: Molecular beacon DNA/protein interaction, on-going.
- Tim Drake**, Ph. D candidate, 2000 - now, **Bioanalytical Chemistry**, Neurotransmission studies using bionanotechnology, on-going.

Marie Vicens, Ph. D candidate, 2000 - now, **Bioanalytical Chemistry**, Molecular design for cancer protein studies, on-going.

## **Undergraduate Students**

**Leng Bang**, 1997-1998, Thesis: Near-field optical probe development and its application in living cells, Graduated with the highest honor from UF, April, 1998.

**Brooks Gross**, 1997-1998, Thesis: Glutamate sensor development for living cell measurements, Graduated with the highest honor from UF, December, 1998.

**Danyell Wilson**, 2001, *NSF Research Experience for Undergraduate program*, Bioanalytical chemistry research: Nanoparticles preparation and bioanalytical application.

**Joshua E. Smith**, 2000, *NSF Research Experience for Undergraduate program*, Bioanalytical chemistry research: Nanoparticles for biomolecular recognition.

**Tiffany Correll**, 1999, *NSF Research Experience for Undergraduate program*, Bioanalytical chemistry research: nanoparticles for DNA/RNA bioanalysis.

**Andres Rojas**, 1999, *NSF Research Experience for Undergraduate program*, Bioanalytical chemistry research: Biomolecular immobilization on solid surface for biosensors.

**Sabine Erhardt**, 1998, *NSF Research Experience for Undergraduate program*, French Student, Bioanalytical chemistry and biophysics research: two-dimensional sensor.

## **Research Associates and Postdoctoral Researchers**

**Dr. Jon Dobson**, 1998, 12 – 1999, 9, **Biomedical engineering and biophysics research**: Optical patch clamp development and two dimensional sensor application (Now a Professor at Department of Biomedical Engineering and Medical Physics at Keele University in England).

**Dr. Xiaohong Fang**, 1998, 4 - 2000, 8, **Bioanalytical chemistry and biophysics research**: Single molecule reaction study, molecular beacon design, immobilization and application, molecular beacon-protein interaction studies (Now a Professor at the Institute of Chemistry, Chinese Academy of Science).

**Dr. Jianwei Jeffery Li**, 1999, 3 – 2002, 7, **Biochemistry and biophysics research**: molecular beacon-protein interaction studies, molecular beacon novel applications, molecular probe design, nanomotor using DNA probes (Now a Research Associate at the Department of Chemistry, Harvard University).

**Dr. Swadeshmukul Santra**, 1998, 11 – 2000, 11, Bioanalytical chemistry and nanotechnology: glutamate chemistry, photophysics and photochemistry of cyclodextrin encapsulated fluorophores, nanoparticle preparation and application (Now an Assistant Professor at the Department of Neuroscience, College of Medicine, University of Florida).

**Dr. Tibor Zelles**, 1997, 1 - 1998, 1, **Neuroscience and biophysics research**: Single cell studies using near-field optics (Now a Professor at the Hungry Academy of Science).

### **High school Students**

**Nathan Williams (High School Student)**, May, 1998 to March, 1999, worked on various tasks in the lab.

**Dillard Woody**, 1998, Bioanalytical chemistry and biophysics research: photopolymerization.

**Alyse Zhang (High School Student)**, 2000, 7 – 2000, 8, worked on protein purification and its interaction with DNA probes.